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(54) Title: METHODS AND COMPOSITIONS FOR PREVENTING THE FORMATION OF ABERRANT RNA DURING TRANSCRIPTION OF A PLASMID SEQUENCE

(57) Abstract: Polynucleotide molecules, which include single stranded DNA or RNA, partially double-stranded DNA, and double-stranded DNA molecules, contain terminator sequences and/or other modifications which suppress the production of unwanted polynucleotide species from these molecules when transfected in a host cell. These molecules are useful in methods for enhancing the efficiency of transcription of a selected polynucleotide sequence in a transfected host cell, and reducing the potential for the products of unwanted transcripts. Further, the methods of the invention are useful in avoiding extinguishing or down regulating the expression of certain polynucleotides present in a host cell or host. These compositions and methods are useful in therapeutic, vaccine, diagnostic and research fields.

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METHODS AND COMPOSITIONS FOR PREVENTING THE  
FORMATION OF ABERRANT RNA DURING TRANSCRIPTION  
OF A PLASMID SEQUENCE

**Field of the Invention**

5           The present invention relates to methods and polynucleotide compositions useful for enhancing the efficiency of expression of polynucleic acid sequences in a host cell by decreasing the expression of undesirable polynucleic acid sequences. More specifically, the invention relates to novel compositions and methods for preventing the expression of aberrant DNA and RNA sequences by use of such compositions.

10          **Background of the Invention**

          Polynucleotide compositions have been described for pharmaceutical uses, primarily for treatment or prophylaxis of disease in mammals, as well as in research in such fields. Specifically a great deal of activity presently surrounds the use of polynucleotide compositions in the treatment of pathogenic extracellular and  
15       intracellular infections, such as viral, bacterial, fungal infections, and the like. As one example, DNA vaccines are described to deliver to a mammalian cell in vivo an agent which will combat a pathogen by harnessing the mammalian immune system. Thus, such vaccines are designed to express, for example, a viral protein or polypeptide, and elicit a humoral or cellular immune response upon challenge by the infective agent.

20       Gene therapy vectors, on the other hand, are polynucleotide compositions generally designed to deliver to a mammalian cell a protein which is either not expressed, expressed improperly or under expressed in a mammal. Such vectors frequently must address species specific immune responses to the those polynucleotide sequences that are recognized as antigenic or which evoke an unwanted cellular immune response.

25       Still other therapeutic uses of polynucleotide compositions are for the delivery of missing or under expressed proteins to a diseased mammalian patient. Furthermore,

polynucleotides are useful themselves as *in vivo* reagents, in diagnostic/imaging protocols, as reagents in gene therapy, in antisense protocols, in vaccine applications or otherwise as pharmaceuticals used to treat or prevent a variety of ailments such as genetic defects, infectious diseases, cancer, and autoimmune diseases. Polynucleotides are also useful as *in vitro* reagents in assays such as biological research assays, medical, diagnostic, screening and contamination detection assays.

A host of problems well-known to the art has prevented the numerous polynucleotide compositions from becoming widely accepted as useful pharmaceuticals. Thus, there are few such DNA vaccines or therapeutics which have yet been accepted by the medical community for the treatment of disease in mammals.

Phenomena have been observed in plants, nematodes, and *Drosophila* that are mediated by polynucleotide compositions, and are referred to as post-transcriptional gene silencing and transcriptional silencing. This phenomenon demonstrates that transfection or infection of a plant, nematode or *Drosophila* with a virus, viroid, plasmid or RNA expressing a polynucleotide sequence having some homology to a regulatory element, such as a promoter or a native gene or a portion thereof already expressed in that cell, can result in the permanent inhibition of expression of both the endogenous regulatory element and/or gene and the exogenous sequence. This silencing effect was shown to be gene specific. See, for example, L. Timmons and A. Fire, Nature, **395**:354 (Oct. 29, 1998); A. Fire *et al*, Nature, **391**:806-810 (Feb. 19, 1998); R. Jorgensen *et al*, Science, **279**:1486-1487 (March 6, 1998); J. R. Kennerdell and r. W. Carthew, Cell, **95**:1017-1026 (Dec. 1998); L. Misquitta and B. M. Paterson, Proc. Natl. Acad. Sci., USA, **96**:1451-1456 (Feb. 1999); M. K. Montgomery *et al*, Proc. Natl. Acad. Sci., USA, **95**:15502-15507 (Dec. 1998)]. A DNA plasmid encoding a full-length pro-alpha 1 collagen gene was transiently transfected into a rodent fibroblast tissue cell line and a "silencing" effect on the native collagen gene and the transiently expressed gene observed [Bahramian and Zarbl, Mol. Cell. Biol., **19**(1):274-283 (Jan. 1999)].

Still another issue with the use of polynucleotide molecules is the formation of aberrant RNA or DNA, instead of the gene-containing transcript intended. The

formation of aberrant RNA or DNA has been postulated to occur in the use of any transfected plasmid or polynucleic acid molecule and to reduce the efficiency of the target gene expression. The formation of aberrant or abnormal RNA can be one cause of the silencing effect noted above.

5           There are available and known in the art many sequences which function to terminate transcription. For example, a pause site for RNA polymerase II is associated with termination of transcription, particularly when the site is positioned immediately downstream of a strong polyadenylation signal in a transient expression system [P. Enriquez-Harris *et al*, EMBO J., 9(7):1833-1842 (1991); G. W. Hatfield *et al*, Mol.  
10 Cell Biol., 3(10):1687-1693 (1983)].

          However, to date, no suggestion has been made to create an effective and functional composition or method to harness termination mechanisms for use in increasing the efficiency of polynucleotide expression in the pharmaceutical, vaccine, gene therapy and diagnostic fields. There exists a need in the art for polynucleotide  
15 compositions and methods of using same to inhibit the formation of aberrant RNA or DNA molecules and increase expression of a selected polynucleotide sequence in a host cell.

### Summary of the Invention

          In one aspect, the invention provides a double stranded polynucleotide  
20 (preferably deoxyribonucleic acid) molecule comprising a first coding strand and a second transcription template strand. The first strand comprises (i) at least one expression cassette sequence which comprises, from 5' to 3', a promoter, a selected polynucleotide sequence the expression of which is controlled by the promoter, and a polyadenylation site, and (ii) at least one first strand terminator sequence. The selected  
25 polynucleotide sequence of the expression cassette can be any polynucleotide sequence which is desired to be expressed in a cell to perform a biological function. The first strand terminator sequence is preferably located 5' to the promoter, located 3' to the polyadenylation site, located on the first strand outside of the expression cassette sequence, or located within the selected polynucleotide sequence of the expression

cassette sequence. The first strand termination is located in a position which does not impede transcription from the sequence on the second strand which is complementary to the expression cassette sequence, nor effect the function of the polynucleotide sequence to be expressed. The second strand is complementary to the first strand. The portions of the second strand sequence complementary to the first strand terminator sequence do not impede transcription from the second strand sequence complementary to the first strand expression cassette. Further, the second strand comprises at least one second strand terminator sequence which terminates transcription initiated on the second strand. The second strand terminator sequence is preferably located on the second strand outside of the sequence complementary to the expression cassette of the first strand and in a position which does not impede transcription from the sequence complementary to the expression cassette sequence, nor impairs the biological function of the polynucleotide sequence, when expressed.

In another aspect, the invention provides a pharmaceutical composition comprising the above-identified double-stranded polynucleotide molecule, an optional agent that facilitates polynucleotide (e.g., DNA) uptake in a cell, and a suitable pharmaceutically acceptable carrier. Such compositions are useful for treating intracellular pathogenic infections, such as viruses. Other such compositions are useful for treating certain cancers. Other such compositions are useful for treating certain extracellular pathogens.

In still another aspect, the invention provides a single stranded polynucleotide sequence selected from a first strand or second strand of a double stranded polynucleotide molecule described above.

In yet another aspect, the invention provides a pharmaceutical composition comprising the above-identified single-stranded polynucleotide molecule, an optional agent that facilitates polynucleotide (e.g., DNA) uptake in a cell, and a suitable pharmaceutically acceptable carrier.

In still another aspect, the invention provides a method for enhancing the efficiency of expression of a selected polynucleotide sequence in a host cell, the method comprising the step of transfecting the host cell with a double stranded DNA

molecule described above or a single strand thereof, and thereby inhibiting the formation of aberrant polynucleotide sequences transcribed from the polynucleotide molecule in the host cell.

5 In another aspect the invention provides a single stranded RNA molecule comprising a sequence of ribonucleic acids having a 5' end and a 3' end, modified to prevent the formation of double-stranded or partially double-stranded regions. In one embodiment, this molecule comprises a 5' cap. In another embodiment, this molecule does not have a cap. In another embodiment, this molecule has a 3' polyA tail. In still another embodiment, this molecule has no polyA tail.

10 In still a further aspect, the invention provides a pharmaceutical composition comprising the above-identified single-stranded RNA molecule, an optional agent that facilitates RNA uptake in a cell, and a suitable pharmaceutically acceptable carrier.

15 In another aspect, the invention provides a method for enhancing the efficiency of expression of a selected polynucleotide sequence in a host cell, the method comprising the step of transfecting the host cell with the single-stranded RNA molecule described above, and thereby inhibiting the formation of aberrant RNA molecules in the host cell.

20 In yet a further aspect, the invention provides a method for treating a mammalian subject comprising administering an effective amount of a pharmaceutical composition comprising any of the polynucleotide molecules described above, an optional agent that facilitates DNA or RNA uptake in a cell, and a suitable pharmaceutically acceptable carrier.

25 In yet another aspect, the invention provides a method for preventing the unintentional shutting off or down-regulation of a polynucleotide sequence in a host cell transfected with a polynucleotide molecule containing a selected polynucleotide sequence homologous to the polynucleotide sequence present in the host cell, the method comprising the steps of : administering an effective amount of a pharmaceutical composition comprising a polynucleotide molecule as described above, an optional agent that facilitates polynucleotide uptake in a cell, and a suitable  
30 pharmaceutically acceptable carrier.

In one embodiment, the polynucleotide compositions or molecules are made by enzymatic synthetic methods or chemical synthetic methods *in vitro*. In another embodiment, the compositions or molecules may be generated in a recombinant culture, e.g., bacterial cells, isolated therefrom, and used in the methods discussed below. In another embodiment the composition generates the polynucleotide molecule *in vivo* after delivery to the host cell.

Still another aspect of the present invention provides such compositions and molecules for use in research methods, such as a reagent for reducing or inhibiting undesired polynucleotide expression in host cells or tissue *in vitro* for use in diagnostic or other research assays, or *ex vivo* for return to the host subject for therapy or other medicinal uses.

Other aspects of the invention are described further in the following detailed description of the preferred embodiments thereof.

#### Brief Description of the Figures

Fig. 1A is an illustration of a double-stranded DNA molecule of this invention containing a first "sense" strand, with the 5' terminator sequence (T<sup>1</sup>), the promoter (P), the selected polynucleotide sequence (PN), the polyadenylation sequence (pA), the 3' terminator sequence (T<sup>2</sup>) and containing a second complementary strand containing the second strand terminator sequence (T<sup>3</sup>), which in this case, is located in a position which is 3' relative to the polyA sequence on the sense strand. Either the first or second strand may be utilized as a single-stranded DNA molecule of this invention.

Fig. 1B is an illustration of another double-stranded DNA molecule of this invention containing a first "sense" strand, with two 5' terminator sequences (T<sup>1</sup> and T<sup>2</sup>), the promoter (P), the selected polynucleotide sequence (PN), the polyadenylation sequence (pA), the 3' terminator sequence (T<sup>3</sup>) and an RNA instability sequence (RIS); and containing a second complementary strand containing multiple second strand terminator sequences (T<sup>4</sup> through T<sup>8</sup>) interspersed in the region which is not complementary to the expression cassette region on the first strand. In this case, terminators T<sup>4</sup> and T<sup>5</sup> are located in positions downstream relative to the polyA

insect promoter [No *et al*, Proc. Natl. Acad. Sci. USA, 93:3346-3351 (1996)], the tetracycline-repressible system [Gossen *et al*, Proc. Natl. Acad. Sci. USA, 89:5547-5551 (1992)], the tetracycline-inducible system [Gossen *et al*, Science, 268:1766-1769 (1995), see also Harvey *et al*, Curr. Opin. Chem. Biol., 2:512-518 (1998)], the  
5 RU486-inducible system [Wang *et al*, Nat. Biotech., 15:239-243 (1997) and Wang *et al*, Gene Ther., 4:432-441 (1997)] and the rapamycin-inducible system [Magari *et al*, J. Clin. Invest., 100:2865-2872 (1997)].

Any component of the expression cassette may be readily selected from among conventionally employed promoters and regulatory sequences used in the  
10 genetic engineering field. See, e.g., Sambrook *et al*, "Molecular Cloning: A Laboratory Manual.", Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press (1989) and other texts in this field. The selection of the expression cassette components are not a limitation of this invention.

Similarly the selected polynucleotide sequence can be any  
15 polynucleotide sequence which encodes a polypeptide, protein, or other product of interest or performs a biological function of interest and is desired to be preferentially and efficiently expressed in a cell *in vitro*, *ex vivo* or *in vivo* for therapeutic use, prophylactic use, gene therapy use, or research or diagnostic use. A host of such selected polynucleotide sequences may be selected by one of skill in the art depending  
20 upon which condition is being treated or prevented. The selection of the polynucleotide sequence will depend upon the use to which the resulting molecule is put. For example, one type of selected polynucleotide sequence includes a reporter sequence, which upon expression produces a detectable signal. Such reporter sequences include without limitation, DNA sequences encoding  $\beta$ -lactamase,  $\beta$ -  
25 galactosidase (LacZ), alkaline phosphatase, thymidine kinase, green fluorescent protein (GFP), chloramphenicol acetyltransferase (CAT), luciferase, membrane bound proteins including, for example, CD2, CD4, CD8, the influenza hemagglutinin protein, and others well known in the art, to which high affinity antibodies directed thereto exist or can be produced by conventional means, and fusion proteins comprising a membrane



bound protein appropriately fused to an antigen tag domain from, among others, hemagglutinin or Myc.

These sequences, when associated with regulatory elements which drive their expression, provide signals detectable by conventional means, including enzymatic, radiographic, colorimetric, fluorescence or other spectrographic assays, fluorescent activating cell sorting assays and immunological assays, including enzyme linked immunosorbent assay (ELISA), radioimmunoassay (RIA) and immunohistochemistry. For example, where the marker sequence is the LacZ gene, the presence of helper virus is detected by assays for beta-galactosidase activity. Where the selected polynucleotide is luciferase, the helper virus may be measured by light production in a luminometer.

However, desirably, the selected polynucleotide is a non-marker sequence encoding a product which is useful in biology and medicine, such as proteins, peptides, anti-sense nucleic acids (e.g., RNAs), enzymes, or catalytic RNAs. The selected polynucleotide may be used to correct or ameliorate gene deficiencies, which may include deficiencies in which normal genes are expressed at less than normal levels or deficiencies in which the functional gene product is not expressed. A preferred type of selected polynucleotide sequence encodes a therapeutic protein or polypeptide which is expressed in a host cell. The invention further includes using multiple selected polynucleotides, e.g., to correct or ameliorate a gene defect caused by a multi-subunit protein. In certain situations, a different selected polynucleotide may be used to encode each subunit of a protein, or to encode different peptides or proteins. This is desirable when the size of the DNA encoding the protein subunit is large, e.g., for an immunoglobulin, the platelet-derived growth factor, or a dystrophin protein. In order for the cell to produce the multi-subunit protein, a cell is infected with the recombinant virus containing each of the different subunits. Alternatively, different subunits of a protein may be encoded by the same selected polynucleotide. In this case, a single selected polynucleotide includes the DNA encoding each of the subunits, with the DNA for each subunit separated by an internal ribozyme entry site (IRES). This is desirable when the size of the DNA encoding each of the subunits is small, e.g., total

of the DNA encoding the subunits and the IRES is less than five kilobases. However, the selected polynucleotide may encode any product desirable for study. The selection of the selected polynucleotide sequence is not a limitation of this invention.

Other useful products encoded by the selected polynucleotide include

5 hormones, growth and differentiation factors including, without limitation, insulin, glucagon, growth hormone (GH), parathyroid hormone (PTH), growth hormone releasing factor (GRF), follicle stimulating hormone (FSH), luteinizing hormone (LH), human chorionic gonadotropin (hCG), vascular endothelial growth factor (VEGF), angiopoietins, angiostatin, granulocyte colony stimulating factor (GCSF),

10 erythropoietin (EPO), connective tissue growth factor (CTGF), basic fibroblast growth factor (bFGF), acidic fibroblast growth factor (aFGF), epidermal growth factor (EGF), transforming growth factor  $\alpha$  (TGF $\alpha$ ), platelet-derived growth factor (PDGF), insulin growth factors I and II (IGF-I and IGF-II), any one of the transforming growth factor  $\beta$  superfamily, including TGF  $\beta$ , activins, inhibins, or any of the bone morphogenic

15 proteins (BMP) BMPs 1-15, any one of the heregulin/neuregulin/ARIA/neu differentiation factor (NDF) family of growth factors, nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophins NT-3 and NT-4/5, ciliary neurotrophic factor (CNTF), glial cell line derived neurotrophic factor (GDNF), neurturin, agrin, any one of the family of semaphorins/collapsins, netrin-1 and netrin-2,

20 hepatocyte growth factor (HGF), ephrins, noggin, sonic hedgehog and tyrosine hydroxylase.

Other useful selected polynucleotide products include proteins that regulate the immune system including, without limitation, cytokines and lymphokines such as thrombopoietin (TPO), interleukins (IL) IL-1 through IL-17, monocyte

25 chemoattractant protein, leukemia inhibitory factor, granulocyte-macrophage colony stimulating factor, Fas ligand, tumor necrosis factors  $\alpha$  and  $\beta$ , interferons  $\alpha$ ,  $\beta$ , and  $\gamma$ , stem cell factor, flk-2/flt3 ligand. Gene products produced by the immune system are also useful in the invention. These include, without limitations, immunoglobulins IgG, IgM, IgA, IgD and IgE, chimeric immunoglobulins, humanized antibodies, single chain

30 antibodies, T cell receptors, chimeric T cell receptors, single chain T cell receptors,

class I and class II MHC molecules, as well as engineered immunoglobulins and MHC molecules. Useful gene products also include complement regulatory proteins such as complement regulatory proteins, membrane cofactor protein (MCP), decay accelerating factor (DAF), CR1, CF2 and CD59.

5                   Still other useful selected polynucleotides may produce a product such as any one of the receptors for the hormones, growth factors, cytokines, lymphokines, regulatory proteins and immune system proteins. The selected polynucleotide may be a receptor for cholesterol regulation, including the low density lipoprotein (LDL) receptor, high density lipoprotein (HDL) receptor, the very low density lipoprotein  
10 (VLDL) receptor, and the scavenger receptor. The selected polynucleotide sequence useful in this invention also encodes products such as members of the steroid hormone receptor superfamily including glucocorticoid receptors and estrogen receptors, Vitamin D receptors and other nuclear receptors. In addition, useful polynucleotide sequences include sequences encoding transcription factors such as *jun*, *fos*, *max*, *mad*,  
15 serum response factor (SRF), AP-1, AP2, *myb*, MyoD and myogenin, ETS-box containing proteins, TFE3, E2F, ATF1, ATF2, ATF3, ATF4, ZF5, NFAT, CREB, HNF-4, C/EBP, SP1, CCAAT-box binding proteins, interferon regulation factor (IRF-1), Wilms tumor protein, ETS-binding protein, STAT, GATA-box binding proteins, e.g., GATA-3, and the forkhead family of winged helix proteins.

20                   Other useful products which may be encoded by the selected polynucleotide sequence of the expression cassette include, carbamoyl synthetase I, ornithine transcarbamylase, arginosuccinate synthetase, arginosuccinate lyase, arginase, fumarylacetic acid hydrolase, phenylalanine hydroxylase, alpha-1 antitrypsin, glucose-6-phosphatase, porphobilinogen deaminase, factor VIII, factor IX, cystathione beta-  
25 synthase, branched chain ketoacid decarboxylase, albumin, isovaleryl-CoA dehydrogenase, propionyl CoA carboxylase, methyl malonyl CoA mutase, glutaryl CoA dehydrogenase, insulin, beta-glucosidase, pyruvate carboxylase, hepatic phosphorylase, phosphorylase kinase, glycine decarboxylase, H-protein, T-protein, a cystic fibrosis transmembrane regulator (CFTR) sequence, and a dystrophin cDNA  
30 sequence.

Still other useful polynucleotide-encoded products include, non-naturally occurring polypeptides, such as chimeric or hybrid polypeptides having a non-naturally occurring amino acid sequence containing insertions, deletions or amino acid substitutions, e.g., single-chain engineered immunoglobulins, antisense molecules and catalytic nucleic acids, such as ribozymes. Still other suitable polynucleotide sequences may be readily selected by one of skill in the art. The selection of the polynucleotide is not considered to be a limitation of this invention. As shown in the following examples, an illustrative selected polynucleotide sequence is the sequence encoding murine interleukin-12.

As part of the first strand are at least one, and desirably at least two, and preferably three or more transcriptional terminator sequences. Transcriptional terminators can be located anywhere on the DNA coding (i.e., the non-transcribed) strand, including within the expression cassette sequence, as long as transcription can proceed uninterrupted within the expression cassette on the DNA template ("second") strand and the biological function of the polynucleotide sequence in the expression cassette is not adversely effected upon expression. Essentially, any modification may be made to the first strand to incorporate the transcription terminators, so long as transcription can take place from the one or more expression cassette complementary region(s) of the second "template" strand and the expressed protein or peptide retains its biological function. Preferred transcription terminators do not create secondary structures that interfere with transcription of the template strand. The function of these terminator sequences is to prevent unwanted transcription from occurring from the first strand, and also from the complementary second transcription template strand, when these strands of the DNA molecule are in the host cell. Importantly, these first strand transcription terminators cannot impede or interrupt the necessary transcription from the second template strand or from an expression cassette intended to be transcribed from the first strand of a bicistronic molecule. For example, the function of these terminator sequences prevents the formation of undesired transcripts which, if homologous to a polynucleotide sequence present in the host cell, could shut off or down regulate its normal transcription in the host cell. Further, the function of these

terminators prevents physical occlusion of the promoter, thereby increasing the efficacy of the expression of the selected polynucleotide. Where the first strand is bicistronic (i.e., contains two or more expression cassettes or each strand contains an expression cassette), the same constraints are placed upon the identity and location of the terminator sequence(s).

Depending upon the number of terminator sequences used in an individual first strand, each terminator sequence may be the same or a different terminator sequence. In one embodiment, a terminator sequence may be a bacterial terminator, such as the 5S ribosomal RNA terminator, *rrnB*. In another embodiment, a terminator sequence may be a bacterial terminator, such as the tryptophan operon terminator, *trpA*. In another embodiment, a terminator sequence may be a bacteriophage terminator, e.g., a lambda 5S RNA terminator or the bacteriophage P1 head protein terminator. Still another desirable terminator sequence which may be employed positioned 3' to a polyA site is a eukaryotic pause site for RNA pol II, such as the site described in P. Enriquez-Harris *et al*, EMBO J., 10(7):1833-1842 (1991). An  $\alpha$ -globin terminator is an example of a terminator for RNA pol II and may also be employed as a terminator sequence in a first strand of this molecule. Further, a histone mRNA processing signal, such as that described in N. Chodchoy *et al*, Mol. Cell. Biol., 11(1):497-509 (Jan. 1991), is a useful terminator sequence for the first strand. Similarly the terminator for the mammalian gastrin gene, the gastrin terminator, is also useful. Another useful terminator sequence is a polynucleic acid sequence that provides a ribozyme cleavage site followed by a pause site terminator sequence, as

“wobbled” codon prevents the transcript made from the DNA molecule in a host cell from undesirably or unintentionally shutting off or down-regulating a homologous polynucleotide sequence which is native to, or essential to, the transfected host cell. See Figs. 2A and 2B. By “substantially all” wobble bases is meant wobbling enough  
5 codons to destroy the homology between the selected polynucleotide and any polynucleotide in the host cell, so as to prevent the shutting off of the polynucleotide sequence in the host cell.

Where the DNA is produced recombinantly, the wobble bases may be altered by well-known mutational techniques. Where the DNA is synthesized, the  
10 wobble codon bases are deliberately made to avoid a match with native sequences in the intended host cell or organism. The wobble bases may be selected to incorporate preference codons to optimize codons for expression in a selected host cell, provided that the aberrant RNA structures discussed above are avoided. See, e.g., United States Patent No. 5,786,464, which teaches the selection of preferred codons.

As used herein and as known in the art, the terms “homology” or  
15 “homologous” refer to the degree of sequence relatedness between two polypeptide or two polynucleotide sequences as determined by the identity of the match between two lengths of such sequences. Both identity and homology can be readily calculated by methods extant in the prior art [See, e.g., COMPUTATIONAL MOLECULAR  
20 BIOLOGY, Lesk, A.M., ed., Oxford University Press, New York, (1988); BIOCOMPUTING: INFORMATICS AND GENOME PROJECTS, Smith, D.W., ed., Academic Press, New York, (1993); COMPUTER ANALYSIS OF SEQUENCE DATA, PART I, Griffin, A.M., and Griffin, H.G., eds., Humana Press, New Jersey, (1994); SEQUENCE ANALYSIS IN MOLECULAR BIOLOGY, von Heinje, G.,  
25 Academic Press, (1987); and SEQUENCE ANALYSIS PRIMER, Gribskov, M. and Devereux, J., eds., M Stockton Press, New York, (1991)]. Methods commonly employed to determine identity or homology between two sequences include, but are not limited to, those disclosed in GUIDE TO HUGE COMPUTERS, Martin J. Bishop, ed., Academic Press, San Diego, 1994, and H. Carillo and D. Lipton, SIAM J. Applied  
30 Math., 48:1073 (1988). Preferred methods to determine identity or homology are

designed to give the largest match between the two sequences tested. Preferred computer program methods to determine identity and homology between two sequences include, but are not limited to, the algorithm BESTFIT from the GCG program package [J. Devereux et al., Nucl. Acids Res., 12(1):387 (1984)], the related  
5 MACVECTOR program (Oxford), and the FASTA (Pearson) programs. The use of such computer programs enable the design of suitable DNA and RNA molecules desired for use in the invention. The algorithm and/or the degree of homology necessary for any particular DNA or RNA molecule may be selected by one of skill in the art. It should be understood that selection of the necessary homology, selection of  
10 the defaults for the program and selection of the program employed to calculate homology is within the skill of the art, given the teachings of this specification and the knowledge extant in the scientific literature.

B. The Second Strand

The double-stranded or partially double-stranded DNA molecule of this  
15 invention, or an alternative single-stranded DNA molecule of this invention, comprises preferably a second "transcription template" strand. While the second strand is ordinarily 100% complementary to the first strand in plasmids produced in bacteria, this is not essential, as, e.g., where at least one of the strands is produced synthetically and combined with a substantially homologous strand. Provided that there is sufficient  
20 homology between the two strands that the  $T_m$  is high enough to cause the strands to stay together under appropriate conditions, certain areas of non-homology with the first strand may be present on the second strand.

The second strand comprises at least one second strand terminator sequence which terminates any transcription initiated on the second strand. The  
25 second strand terminator sequence is located on the second strand in a sequence of the second strand which is not complementary to the expression cassette sequence. Preferably, the second strand of the DNA molecule contains more than one second strand terminator sequence. More preferably, the second strand contains from two terminator sequences to one terminator sequence for every 100 nucleotides of second  
30 strand outside of the sequence complementary to the expression cassette. Another

preferred embodiment contains one terminator sequence for every 500 nucleotides of second strand in the region which is not complementary to the expression cassette of the first strand. If more than one second strand terminator sequence is used, the multiple terminator sequences may be interspersed in the region of the second strand which is not complementary to the expression cassette, as described above.

In another embodiment, where it is desirable to include a terminator sequence at one or more positions on the first sense strand, including within the cistron or expression cassette, even within the gene or polynucleotide of interest, the second antisense DNA strand may have some degree of non-homology which needs to be transcribed into the mRNA. Still another embodiment is a second strand that contains one or more transcription terminators or RNA instability sequences outside of the region complementary to the coding region on the first strand. Changes in the second antisense strand can ordinarily be made only insofar as such changes will nevertheless yield a functional polypeptide and will not adversely effect transcription. While *cis* terminator or instability sequences may be encoded within the sense strand of a plasmid, nucleotides on the second, antisense or transcribed strand must include only conservative or nonsense mutations or other mutations which do not change or do not adversely affect the resulting amino acid sequence. In such embodiments, the function of any expressed protein must not be adversely affected.

Each terminator sequence on the second strand may be the same or each may each be a different terminator sequence. These terminator sequences include the sequences identified above as first strand terminator sequences. In one embodiment, a terminator sequence may be a bacterial terminator, such as the *rrnB* or *trpA*. In another embodiment, a terminator sequence may be a bacteriophage terminator, e.g., a lambda 5S RNA terminator or the bacteriophage P1 head protein terminator. Still another desirable terminator sequence which may be employed is a polynucleic acid sequence that provides a ribozyme cleavage site or a ribonucleic acid cleavage site, followed 3' by a eukaryotic pause site, e.g., a pause site for RNA pol II or an  $\alpha$ -globin terminator. Further, a histone mRNA processing signal is a useful terminator sequence for the second strand. Similarly the terminator for the mammalian



gastrin gene, the gastrin terminator, is also useful. Both rho-dependent terminators and rho-independent terminators are also sequences which may be employed for this purpose in the second strand. Additionally, a padlock terminator sequence as described above is a useful terminator for the second strand if it is part of a double-stranded or partially double-stranded DNA molecule.

In one embodiment of a second strand of this invention, the terminator sequence is located in the region of the second strand which is not complementary to the expression cassette, and is positioned immediately 3' with reference to the first strand poly A site (see Fig. 1B). In another embodiment of this invention, a second strand terminator sequence is located less than 200 nucleotides from the poly A site in the region of the second strand which is not complementary to the expression cassette sequence (see Fig. 1C). An alternative embodiment of the second strand contains a terminator sequence is located 5' with reference to the first strand promoter sequence (see Figs. 1A-1C). A preferred embodiment contains multiple terminator sequences interspersed throughout the region of the second strand which is not complementary to the expression cassette of the first strand (see Figs. 1B and 1C).

Additional embodiments of the second strand of the DNA molecule involve the second strand additionally containing at least one RNA instability sequence located on the region of the second strand sequence which is not complementary to the expression cassette sequence. Preferably, more than a single instability sequence is present in that region of the second strand. Further, as with the first strand, the second strand preferably does not contain any complementary inverted repeat sequence of greater than seven nucleotides for use in mammalian cells or mammals or four nucleotides for use in invertebrate cells or organisms.

As another embodiment of a second strand of a DNA molecule of this invention, the "wobble" nucleotides in the second strand can be manipulated to alter the nucleotide sequence and prevent or eliminate any inverted complementary repeats in the sequence. This manner of eliminating such repeats permits the DNA to avoid being transcribed into RNA that will form double-stranded regions. Alternatively, an embodiment of the second strand of the DNA molecule of this invention, involves

altering the wobble nucleotides in substantially all of the codons in that portion of the second strand that is complementary to the selected polynucleotide sequence, where there is a possibility that the selected polynucleotide sequence in the molecule is too homologous with a polynucleotide sequence already present in the host cell. By  
5 altering only those bases which change the codon without changing the encoded amino acid, the selected polynucleotide sequence thus encodes the same amino acid sequence, but provides a nucleotide sequence which is substantially nonhomologous to a desired polynucleotide sequence in a host cell or host organism. This area of non-complementarity in the "wobbled" codon prevents the transcript made from the DNA  
10 molecule in a host cell from undesirably or unintentionally shutting off or down-regulating a homologous polynucleotide sequence which is native to, or essential to, the transfected host cell. See Figs. 2A and 2B. By "substantially all" wobble bases is meant wobbling enough codons to destroy the homology between the selected polynucleotide and any polynucleotide in the host cell, so as to prevent the shutting off  
15 of the polynucleotide sequence in the host cell. Where the DNA is produced recombinantly, the wobble bases may be altered by well-known mutational techniques. Where the DNA is synthesized, the wobble bases are deliberately made to avoid a match with native sequences in the intended host cell or organism.

The DNA molecules of this invention, whether double-stranded or  
20 partially double-stranded, and comprising a first and a second strand as described above, or a single strand, formed of either the first or the second strand as described above, permits the nucleotide sequence which is transcribed within the cell to become a single stranded RNA sense or anti-sense strand which lacks the capacity to form any significant double strandedness. The DNA molecule can provide a single stranded  
25 RNA sequence comprising both a sense polynucleotide sequence and an anti-sense polynucleotide sequence, optionally separated by a non-base paired polynucleotide sequence.

These DNA molecules of the invention may be prepared and used in the methods described in detail below.

formulation for efficacy in the target human or animal. Suitable exemplary preservatives include chlorobutanol, potassium sorbate, sorbic acid, sulfur dioxide, propyl gallate, the parabens, ethyl vanillin, glycerin, phenol, and parachlorophenol. Suitable stabilizing ingredients which may be used include, for example, casamino  
5 acids, sucrose, gelatin, phenol red, N-Z amine, monopotassium diphosphate, lactose, lactalbumin hydrolysate, and dried milk. A conventional adjuvant is used to attract leukocytes or enhance an immune response. Such adjuvants include, among others, Ribi, mineral oil and water, aluminum hydroxide, Amphigen, Avridine, L121/squalene, D-lactide-poly(lactide)/glycoside, pluronic polyols, muramyl dipeptide, killed *Bordetella*,  
10 and saponins, such as Quil A.

In addition, other agents which may function as transfecting agents and/or replicating agents and/or inflammatory agents and which may be co-administered with the composition of this invention, include growth factors, cytokines and lymphokines such as alpha-interferon, gamma-interferon, platelet derived growth factor (PDGF),  
15 colony stimulating factors, such as G-CSF, GM-CSF, tumor necrosis factor (TNF), epidermal growth factor (EGF), and the interleukins, such as IL-1, IL-2, IL-4, IL-6, IL-8, IL-10 and IL-12. Further, fibroblast growth factor, surface active agents such as immune-stimulating complexes (ISCOMS), Freund's incomplete adjuvant, LPS analog including monophosphoryl Lipid A (MPL), muramyl peptides, quinone analogs and  
20 vesicular complexes such as squalene and hyaluronic acid may also be administered in conjunction with the compositions of the invention.

The pharmaceutical compositions may also contain other additives suitable for the selected mode of administration of the composition. Thus, these compositions can contain additives suitable for administration via any conventional route of  
25 administration, including without limitation, parenteral administration, intraperitoneal administration, intravenous administration, intramuscular administration, subcutaneous administration, intradermal administration, oral administration, topical administration, intranasal administration, intra-pulmonary administration, rectal administration, vaginal administration, and the like. All such routes are suitable for administration of these

compositions, and may be selected depending on the agent used, patient and condition treated, and similar factors by an attending physician.

The composition of the invention may also involve lyophilized polynucleotides, which can be used with other pharmaceutically acceptable excipients for developing powder, liquid or suspension dosage forms, including those for intranasal or pulmonary applications. See, e.g., Remington: The Science and Practice of Pharmacy, Vol. 2, 19<sup>th</sup> edition (1995), e.g., Chapter 95 Aerosols; and International Patent Application No. PCT/US99/05547, the teachings of which are hereby incorporated by reference. Routes of administration for these compositions may be combined, if desired, or adjusted.

In some preferred embodiments, the pharmaceutical compositions of the invention are prepared for administration to mammalian subjects in the form of, for example, liquids, powders, aerosols, tablets, capsules, enteric coated tablets or capsules, or suppositories.

The compositions of the present invention, when used as pharmaceutical compositions, can comprise about 1 ng to about 20 mgs of polynucleotide molecules e.g., the synthetic RNA molecules or DNA molecules, plasmids, viral vectors, recombinant viruses, and mixtures thereof. In some preferred embodiments, the compositions contain about 10 ng to about 10 mgs of polynucleotide sequences. In other embodiments, the pharmaceutical compositions contain about 0.1 to about 500  $\mu$ g polynucleotide sequences. In some preferred embodiments, the compositions contain about 1 to about 350  $\mu$ g polynucleotide sequences. In still other preferred embodiments, the pharmaceutical compositions contain about 25 to about 250  $\mu$ g of the polynucleotide sequences. In some preferred embodiments, the vaccines and therapeutics contain about 100  $\mu$ g of the polynucleotide sequences.

The compositions of the present invention in which the DNA or RNA molecules are delivered in donor cells or bacterium can be delivered in dosages of between about 1 cell to about  $10^7$  cells/dose. Similarly, where the delivery agent is a live recombinant virus, a suitable vector-based composition contains between  $1 \times 10^2$  pfu to  $1 \times 10^{12}$  pfu per dose.

The above dosage ranges are guidelines only. In general, the pharmaceutical compositions are administered in an amount effective to treat or prevent the diseases, disorders or infections for which it is designed. The amount of the pharmaceutical composition in a dosage unit employed will be determined empirically, based on the response of cells *in vitro* and response of experimental animals to the compositions of this invention. It will be appreciated that optimum dosage will be determined by standard methods for each treatment modality and indication. Thus the dose, timing, route of administration, and need for readministration of these compositions may be determined by one of skill in the art, taking into account the condition being treated, its severity, complicating conditions, and such factors as the age, and physical condition of the mammalian subject, the employment of other active compounds, and the like.

Still another embodiment of this invention is the use of the DNA and RNA molecules of this invention to prevent the unintentional or undesirable shutting off or down regulating of polynucleotide sequences which are essential or desirable in a host cell transfected with a polynucleotide molecule containing a polynucleotide sequence homologous to a polynucleotide sequence native to the host cell. This method comprises the steps of administering an effective amount of a pharmaceutical composition comprising a polynucleotide molecule as described above, an optional agent that facilitates polynucleotide uptake in a cell, and a suitable pharmaceutically acceptable carrier.

Depending upon the identity of the selected polynucleotide sequence in the molecules described above, the compositions, pharmaceutical compositions, dosages and modes of administration described above are particularly desirable for the treatment of a variety of disorders that plague vertebrates, especially mammals, but also avians and other fowl, as well as invertebrates, such as fish, including infections by heterologous pathogenic organisms, either extracellular or intracellular pathogens. Additionally, the compositions of this invention are useful in preventing infection of a host with a pathogen, or in treating cancers. Further, these compositions are useful in the treatment of inherited or genetic disorders by expressing efficiently a polynucleotide protein or function which the host lacks.

One of skill in the art, given this disclosure can readily select viral families and genera, or pathogens including prokaryotic and eukaryotic protozoan pathogens as well as multicellular parasites, for which therapeutic or prophylactic compositions according to the present invention can be made. See, e.g., the tables of such pathogens in general immunology texts and in U. S. Patent No. 5,593,972, incorporated by reference herein. One of skill in the art can readily select disorders described above, and can also readily design the appropriate selected polynucleotide sequences for use in treatment, or prophylaxis of a disease.

#### *V. Other Methods of The Present Invention*

The compositions described above, and the general methods of using these compositions to enhance the expression of a selected polynucleotide sequence in a host cell and thereby reduce the production or transcription or aberrant sequences can also be applied to a variety of research, and *in vitro* applications, where such efficient expression is essential. These compositions and methods may also be applied to manipulate plant cells and insect cells, and the hosts may similarly be plants and insects, among the other organisms mentioned previously.

Similarly, application of this method can be used to make cell lines of mammalian, bacterial, yeast, fungal, insect, plant, and other origins which efficiently produce a selected polynucleotide product. These molecules may also be employed to increase the efficiency of stable cell lines used to produce certain polynucleotide-encoded products. Such manipulated cells may be employed in producing recombinant proteins for pharmaceutical use in animals, for vaccine use in animals, as well as for a variety of uses in the agricultural fields. Such cells may also be employed in conventional testing assays for drugs or other useful compounds, or for drug screening and development assays, etc. Still other uses are expected to be obvious to the person of skill in the art given the teachings herein.

The following examples illustrate methods for preparing the compositions and using the compositions of this invention to increase the efficiency of transcription and thus expression of a selected polynucleotide sequence, and to reduce or inhibit

expression of aberrant (unintended) polynucleotide sequences. It is understood by one of skill in the art, that other selections for the various selected polynucleotides of the compositions, and RNA and/or DNA molecules may be readily made as taught by this specification. These examples are illustrative only and do not limit the scope of the invention.

EXAMPLE 1: A DNA Plasmid Containing Terminator Sequences on the Coding Strand using Mouse Interleukin 12 (mIL12) p40 as the Selected Polynucleotide

Experiments with expression of murine IL12 p40 from DNA plasmid constructs have been reported in literature. In the absence of an immune response (non-antigenic in mouse), the expression of the IL12 p40 protein in serum peaks at day 8, and decreases to basal (endogenous) levels by day 50.

In the following example, the plasmid sequence is modified by the insertion of terminator sequences to prevent the formation of aberrant RNA. The cloning sites in the mIL12 expression vector are shown in Fig. 6, as a HpaI site at nt#4700 (downstream of the polyA site), and an SphI site at nt#2568 (upstream of the CMV promoter/enhancer/ RSV enhancer module). The plasmid encoding mIL12 (Fig. 6) is modified to contain sequences (elements) that cause termination of elongation. The terminator sequences identified below are placed upstream to the human CMV promoter at the promoter cloning site, and at about 150 bases downstream of the SV40 polyadenylation sequence. When the terminator is used upstream of the promoter, a polyadenylation sequence is also cloned 150 base pairs upstream of the terminator sequence, since some termination sequences efficiently perform only when associated with a polyA site.

The terminator sequences inserted into the plasmids include the following: Bacterial polymerase terminators which are *rho* dependent and *rho* independent are *trpA* (Genbank accession #E02304) and *rmBT1T2* (Amersham-Pharmacia Biotech catalog #27-4925-01, Brosius, J., Gene, 27:151 (1984), Genbank accession #U13859), respectively. These sequences are isolated from existing vectors available from Pharmacia Biotech or these sequences are readily synthesized (Genbank accession

bacterial terminator, a bacteriophage terminator, a sequence comprising a ribozyme cleavage site or a ribonucleic acid cleavage site followed by a eucaryotic RNA pause site or an  $\alpha$ -globin terminator, a histone processing signal, a rho-dependent terminator, and a rho-independent terminator.

35. A pharmaceutical composition comprising a polynucleotide molecule of any of claims 33-34, an optional agent that facilitates polynucleotide uptake in a cell, and a suitable pharmaceutically acceptable carrier.

36. A substantially single-stranded RNA molecule comprising a 5' end, a ribonucleotide sequence having a selected biological function when translated in a host cell, and a 3' end, said molecule being incapable of stably forming a double-stranded or partially double-stranded RNA molecule.

37. The molecule according to claim 36 wherein wobble nucleotides are altered to prevent the occurrence of an inverted complementary repeat.

38. The molecule according to claim 36, modified to prevent the extension of a hairpin at the 3' end.

39. The molecule according to claim 36 wherein said molecule contains no inverted complementary repeat sequences of greater than 7 nucleotides in length.

40. The molecule according to claim 36 wherein said molecule contains no inverted complementary repeat sequences of greater than 4 nucleotides in length.

41. The molecule according to claim 36 which comprises a cap at the 5' end of said molecule.



42. The molecule according to claim 38 wherein said modification comprises attached a chain terminator at the 3' end of said sequence.

43. The molecule according to claim 36 which comprises a Kozak sequence positioned in said sequence 5' to the 5' codon.

44. The molecule according to claim 36 which comprises a polyA tail.

45. The molecule according to claim 36 which comprises no polyA tail.

46. A substantially single-stranded RNA molecule wherein wobble bases in substantially all codons in the portion of the molecule sequence that comprises a selected polynucleotide sequence are modified to encode the same amino acid sequence, but provide a nucleotide sequence which is substantially nonhomologous to a polynucleotide sequence present in a host cell.

47. A pharmaceutical composition comprising a polynucleotide molecule of any of claims 36-45, an optional agent that facilitates RNA uptake in a cell, and a suitable pharmaceutically acceptable carrier.

48. A method for enhancing the efficiency of expression of a selected polynucleotide sequence in a host cell, said method comprising the step of transfecting said host cell with a double stranded polynucleotide molecule comprising a first coding strand and a second transcription template strand,

(a) said first coding strand comprising

(i) an expression cassette sequence which comprises, from 5' to 3', a promoter, a selected polynucleotide sequence the expression of which is controlled by said promoter, and a polyadenylation site, and

(ii) at least one first strand terminator sequence; and

(b) said second strand complementary to said first strand, wherein the portions of said second strand sequence complementary to the first strand terminator sequence do not impede transcription from the second strand sequence complementary to said first strand expression cassette, and wherein said second strand comprises at least one second strand terminator sequence which terminates transcription initiated on said second strand outside of the second strand sequence that is complementary to said first strand expression cassette,

thereby inhibiting the formation of aberrant polynucleotide sequences transcribed from said polynucleotide molecule in said host cell.

49. The method according to claim 48, wherein said first strand terminator sequence is selected from the group consisting of a terminator sequence located 5' to said promoter, a terminator sequence located 3' to said polyadenylation site, a terminator sequence located on said first strand outside of said expression cassette sequence, and a terminator sequence located within the selected polynucleotide sequence of said expression cassette sequence.

50. The method according to claim 48, wherein said second strand terminator sequence is located on said second strand in a sequence which is not complementary to said first strand expression cassette sequence.

51. A method for treating a host subject comprising administering an effective amount of a pharmaceutical composition comprising a polynucleotide molecule of any of claims 1-31, an optional agent that facilitates polynucleotide uptake in a cell, and a suitable pharmaceutically acceptable carrier.

52. A method for enhancing the efficiency of expression of a selected polynucleotide sequence in a host cell, said method comprising the step of transfecting said host cell with a substantially single-stranded RNA molecule comprising a 5' end, a ribonucleotide sequence having a selected biological function when translated in a host cell, and a 3' end, said molecule being incapable of stably forming a double-stranded or partially double-stranded RNA molecule.

53. A method for treating a host subject comprising administering an effective amount of a pharmaceutical composition comprising a polynucleotide molecule of any of claims 36 through 45, an optional agent that facilitates RNA uptake in a cell, and a suitable pharmaceutically acceptable carrier.

54. A method for preventing the inadvertent shutting off or down regulation of a polynucleotide sequence present in a host cell transfected with a polynucleotide molecule containing a polynucleotide sequence homologous to said polynucleotide sequence, said method comprising the steps of :

administering an effective amount of a pharmaceutical composition comprising a polynucleotide molecule of any of claims 1 through 31 and 36 through 45, an optional agent that facilitates polynucleotide uptake in a cell, and a suitable pharmaceutically acceptable carrier.

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FIG. 1A

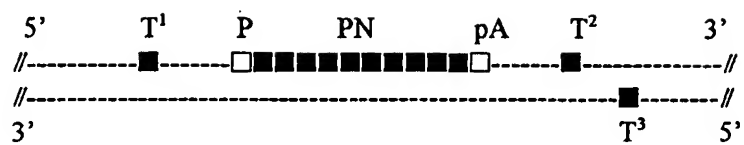


FIG. 1B

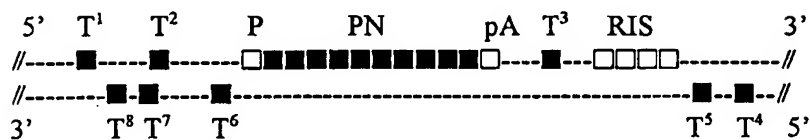


FIG. 1C

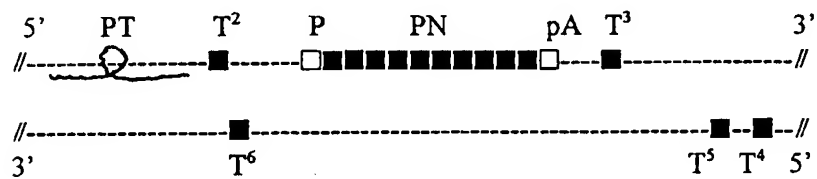


FIG. 1D

